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# Cigarette smoke extract induces differential expression levels of beta-defensin peptides in human alveolar epithelial cells

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## Abstract

**Background:** The damaging effects of cigarette smoke on the lungs are well known in terms of cancer risks. Additional molecular changes within the lung tissue can also occur as a result of exposure to cigarette smoke. The human  $\beta$ -defensin (hBD) class of antimicrobial peptides is the focus of our research. In addition to antimicrobial activity,  $\beta$ -defensins also have immunomodulatory functions. Over 30 previously unrecognized  $\beta$ -defensin genes have recently been identified in the human genome, many with yet to be determined functions. We postulated that altered  $\beta$ -defensin production may play a role in the pathogenesis observed in the lungs of smokers. Our hypothesis is that cigarette smoke exposure will affect the expression of  $\beta$ -defensins in human lung alveolar epithelial cells (A549).

**Methods:** We exposed A549 cells to cigarette smoke extract (CSE) and measured the changes in mRNA levels of several antimicrobial peptides by quantitative real-time PCR, and directly observed peptide expression in cells by immunofluorescence (IF) microscopy.

**Results:** We found that hBD3, hBD5, and hBD9 gene expression was upregulated in A549 cells exposed to CSE. HBD1, hBD8, hBD18 and LL-37 gene expression did not significantly change upon exposure to CSE. Expression of hBD3 and hBD4 peptides was visualized by IF.

**Conclusions:** This differential expression suggests that hBD3, hBD5, and hBD9 may play a role in the changes to the lung tissue observed in smokers. Establishing differential  $\beta$ -defensin expression following CSE treatment will add to our understanding of the molecular response of the lung alveolar epithelium to cigarette smoke exposure.

**Keywords:** Cigarette smoke extract,  $\beta$ -defensins, Lung, Alveolar epithelial cells, Antimicrobial peptide

## Background

Smoking cigarettes increases the risk of contracting lung cancer [1]. Lung cancer caused an estimated 159,300 deaths in the United States in 2009, and is the leading cause of cancer related mortality [2]. In addition to causing cancer, smoking increases the risk of dying prematurely due to chronic obstructive pulmonary disease (COPD) or emphysema [3], stroke and cardiac arrest [4]. Cigarette smoke contains thousands of chemical components, many of which are capable of acting as either carcinogens [5] or

foreign antigens [6-8]. Once inhaled, some cigarette smoke components adhere to cells lining the respiratory tract, triggering a robust response by lung cells, including activating the innate immune system [9].

Antimicrobial Peptides (AMPs) are small (<100 amino acids) peptides that play a critical role in host airway defense as part of the innate immune system [10].  $\alpha$  and  $\beta$  are the two major classes of defensin AMPs.  $\beta$ -defensin expression is associated with some chronic lung diseases, including COPD [11-14]. In addition to  $\alpha$  and  $\beta$  defensins, humans possess one member of the cathelicidin class of AMP, LL-37, which was also assessed in these experiments.  $\alpha$ -defensins are not expressed in epithelial cells, and were not measured in the present study.

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The Human Lung Alveolar Epithelial (A549) cell line is a model system used to model alveolar epithelial type II cells (ATII). We used this model for the quantification of relative AMP expression levels after exposure to bacteria [15]. Although this model has been extensively used in the literature, the limitation of this model is that it does not undergo differentiation to express the alveolar type I (ATI) phenotype [16]. Given the specific goal of this experiment, we determined that the A549 model was an appropriate tool for our study. In this experiment we tested mRNA level changes in response to exposure to cigarette-smoke extract (CSE) of known as well as recently identified  $\beta$ -defensin genes [17-20].

Until recently, only four human  $\beta$ -defensins (hBDs) were known to be expressed in respiratory epithelial cells (hBD1, hBD2, hBD3 and hBD4) [21-23]. However, recent computational and genomic research has predicted over 30 additional human  $\beta$ -defensins, many of which have not been investigated for their biological function [17,24]. Importantly, defensin peptides appear to play multiple roles *in vivo* and exhibit an immunomodulatory function, similar to cytokines or chemokines. Expression of  $\beta$ -defensins can be induced via NF- $\kappa$ B activation mainly through TLR receptor binding to microbial components, or via pro-inflammatory cytokines, such as TNF- $\alpha$  and IL1- $\beta$ . Weinberg characterized  $\beta$ -defensins as complex molecules whose proper function is critical for effective immune system function and that when  $\beta$ -defensin production is altered, especially in areas where cells are already not functioning properly, AMP expression may actually increase disease progression [25]. In addition to the pathological changes observed in lungs exposed to smoke, exposed bronchial epithelial cells secrete many cytokines with the ability to stimulate immune cell migration [26].  $\beta$ -defensin expression may also produce a cellular environment where inflammation is enhanced [25] and vascular permeability is increased [27].

Recent publications have shown that exposure of cells to cigarette smoke represses the induction of hBD2 expression by cells exposed to the bacterial lipopolysaccharide [28,29]. Human gingival epithelial cells exposed to cigarette smoke show increased expression of several  $\beta$ -defensins as well as proinflammatory cytokines [30]. Cigarette smoke directly or indirectly alters the expression of hBD2 [31]. It has also been suggested that this peptide may play a role in the pathogenic effects caused by cigarette smoke [20]. Our hypothesis is that exposure of Human Alveolar Epithelial Cells (A549) to cigarette smoke will lead to measurable changes in gene expression levels for some of the newly-identified human  $\beta$ -defensin peptides (hBDs) as measured by quantitative real-time PCR (qRT-PCR). Our finding that exposure to cigarette smoke increases certain  $\beta$ -defensin gene expression would suggest that repeated exposure with each new cigarette

would lead to chronic over-expression of those  $\beta$ -defensins and possibly exacerbate the deleterious effects of cigarette smoke-damaged cells.

## Materials and methods

### $\beta$ -defensin induction in A549 cells

Low passage human lung epithelial cell line (A549, ATCC CCL-185) were grown to 95% confluence in 6 well culture plates ( $1 \times 10^6$  cells per well, BD catalog # 353046) with Ham's F-12 (Cellgro 10-080-CV) containing 10% heat inactivated fetal bovine serum, 37°C, 5% CO<sub>2</sub>. Cells were then washed with PBS and fed serum-free Hams F-12 media overnight. Following the previously established protocol [5,31], cells were exposed to either 50  $\mu$ g/ml of the 40 mg/ml stock solution of cigarette smoke extract (CSE in 100% DMSO) in Ham's F-12 media, or an equivalent amount of DMSO alone in Ham's F-12 media, or 10 ng/ml of IL-1 $\beta$  (Sigma) in Ham's F-12 media, for 30 minutes.

### Cigarette Smoke Extract (CSE)/Cigarette smoke condensate (CSC)

CSE was purchased from Murty Pharmaceuticals, Lexington KY [5,31]. According to the manufacturer CSE was produced by burning one University of Kentucky "1R13" cigarette and extracting the total particulate into 100% DMSO to prepare a 40 mg/ml stock solution. Cells were exposed to 50  $\mu$ g/ml of the 40 mg/ml stock solution of cigarette smoke extract (CSE in 100% DMSO) suspended in Ham's F-12 media. The control cells received the equivalent volume of DMSO devoid of CSE suspended in Ham's F-12 media. Previously published reports using this material were followed [5,31].

### Analysis of AMP gene expression by quantitative reverse transcription qRT-PCR

After exposure, cells were harvested, mRNA, extracted, and cDNA made following our previously published protocol for qRT-PCR of antimicrobial peptides [15,32]. Quantitative RT-PCR was performed with primers specific for the following  $\beta$ -defensin peptides: hBD1, hBD3, hBD5, hBD6, hBD8, hBD9, hBD18, and the cathelicidin LL-37 in order to measure how much each gene was activated or inhibited by the cigarette smoke treatment. Control cells were used as a baseline to compare induction of gene expression in cells exposed to cigarette smoke extract, and levels of 18S rRNA were used to normalize between samples, leading to the relative units of expression indicated for each experiment. Using a previously established protocol [15,32], quantitative real-time PCR analysis in a MyiQ Single Color Real-Time PCR Detection System (BioRad Laboratories) was performed according to the manufacturer's instructions. Primer sequences and melting temperatures are summarized in Table 1.

**Table 1 Quantitative RT-PCR primer sequences used in this study**

Primers	Sequences	Melting temp	Product size	Reference
HBD1 Forward	5'-CCCAGTTCCTGAAATCCTGA-3'	56°C	215bp	(Han et al., 2008) [15]
Reverse	5'-CAGGTGCCTTGAATTTGGT-3'	56°C		
HBD3 Forward	5'-AGCCTAGCAGCTATGAGGATC-3'	56°C	206bp	(Han et al., 2008) [15]
Reverse	5'-CTTCGGCAGCATTTTGCGCCA-3'			
HBD5 Forward	5'-TCCATCAGGTGAGTTTGCTG-3'	57°C	105bp	(*)
Reverse	5'-GTTCAGCCTGCAATTTCCAT-3'			
HBD8 Forward	5'-TGCCTTGAACAGAAATCCA-3'	56°C	104bp	(**)
Reverse	5'-TCCTTTTGGGTGTAGTGCTC-3'			
HBD9 Forward	5'-GGCCTAAATCCAGGTGTGAA-3'	57°C	174bp	(Alekseeva et al., 2009) [33]
Reverse	5'-TCAAATGTTGGCAAGTGGAG-3'			
HBD18 Forward	5'-TGCATTCCATCCAATGAAGA-3'	57°C	181bp	(***)
Reverse	5'-GAGGTCTCAGTCCCCTTCC-3'			
LL-37 Forward	5'-CTAGAGGGAGGCAGACATGG-3'	57°C	201bp	(Amer, Bishop, & van Hoek, 2010) [32]
Reverse	5'-AGGAGGCGGTAAGGTTAGC-3'			

This table summarizes the primers that were used in this study, the melting temperature, the predicted product size and relevant references. Invitrogen OligoPerfect™ Designer was used to design new primers. (\*) NM\_152250.1 Homo sapiens defensin, beta 105A (DEFB105A), mRNA; (\*\*) NM\_001002035.1 Homo sapiens defensin, beta 108B (DEFB108B), mRNA; (\*\*\*) NM\_054112.2 Homo sapiens defensin, beta 118 (DEFB118), mRNA.

### Immunofluorescence

A549 cells were grown on chambered slides (BD Falcon, 354108) and exposed to CSE as above for  $\beta$ -defensin induction. Cells were fixed, permeabilized and blocked as previously described [34], and a 1:500 dilution of anti-human  $\beta$ -defensin primary antibody (Abcam, ab14421 Anti-beta Defensin 1 antibody, ab19270 Anti-beta 3 Defensin antibody, ab70215 Anti-beta 4 Defensin antibody) was applied for 1 hr. Detection was performed with 1:5000 dilution of secondary antibody labeled with Alexafluor 488 (Green), using ProLong Gold anti-fade with DAPI (Blue). Images were obtained as previously described [34], using the Nikon Confocal microscope.

### Statistical analysis

Statistical analysis and graphing was carried out using Prism 5 software (GraphPad, La Jolla, CA). The two-tailed t-test assuming unequal variance was used to compare negative control  $\beta$ -defensin gene expression of the two different experimental groups expression profiles (IL-1 $\beta$ , cigarette smoke extract, CSE) compared to the control (DMSO alone).

### Ethical & safety approvals

All experiments were approved by the Institutional Review Board at George Mason University. Since only a commercially available, de-identified human cell line was used, no ethical approvals were required for this research.

### Results

Based on emerging reports of potentially important roles for some of the newly discovered human  $\beta$ -defensins

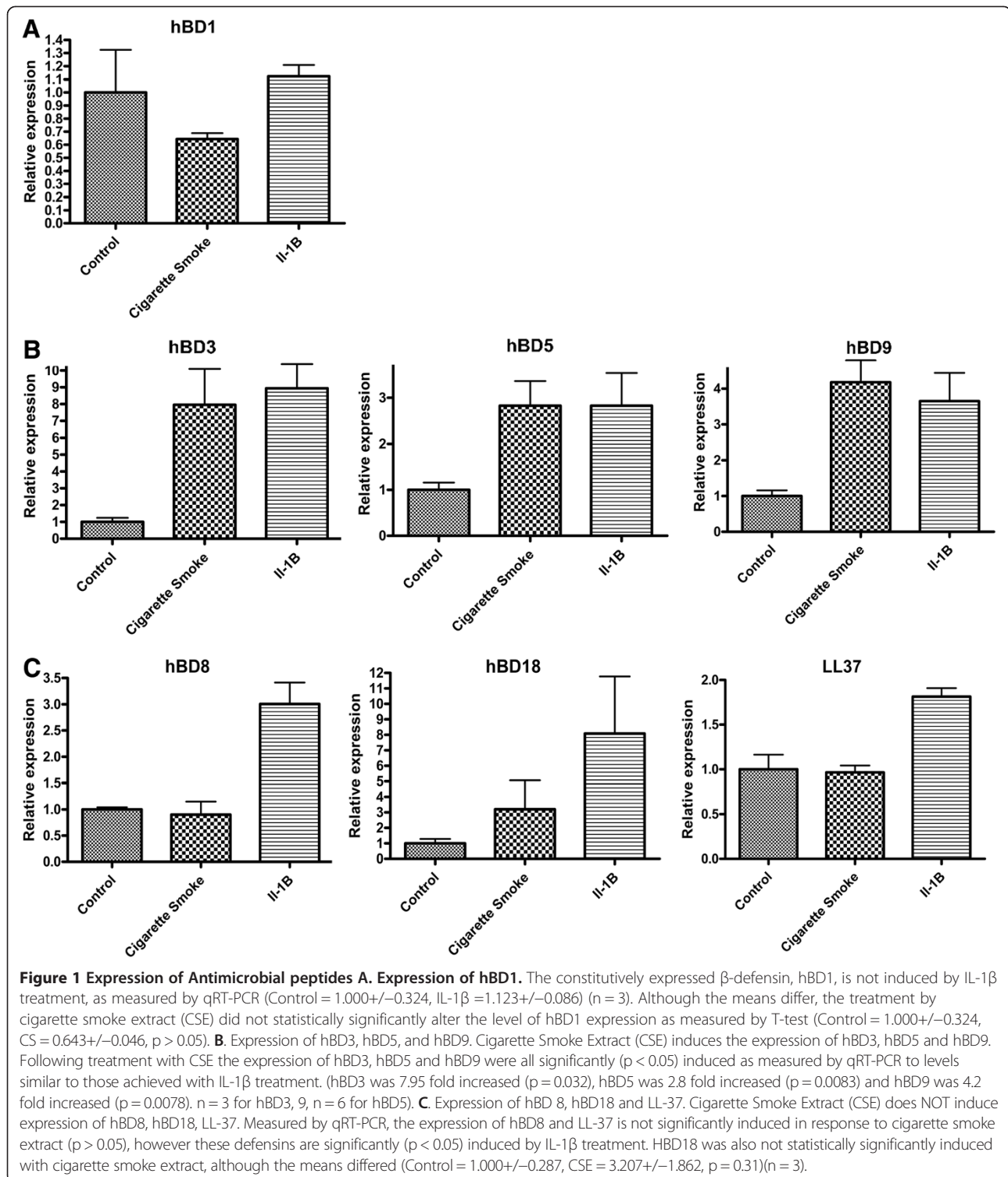
in lung defense (hBD3, hBD5, hBD8, hBD9, hBD18) [18,24,33], and the emerging role of hBD1 in COPD [11], we chose to study this selected set of the human  $\beta$ -defensin genes that had not been previously examined for their alteration following exposure to cigarette smoke (CSE) [29,35]. We included the human cathelicidin, LL-37/CAMP, as a comparator, as it also is thought to play a significant role in lung defense [17].

### HBD1

The first identified human  $\beta$ -defensin gene, hBD1, is considered a constitutively produced antimicrobial peptide, whose expression has not been observed to change significantly under physiological conditions [36,37]. Following treatment with 50  $\mu$ g/ml cigarette smoke (CSE), there is no significant change in hBD1 expression (Control = 1.000 $\pm$ 0.324, CSE = 0.643 $\pm$ 0.046,  $p > 0.05$ ) compared to mock treated cells. This confirms previous findings, as the expression of this peptide is also not up-regulated by 10 ng/ml of IL-1 $\beta$  treatment (IL-1 $\beta$  = 1.123 $\pm$ 0.086) ( $n = 3$ ) compared to mock treated cells (Figure 1A).

### HBD3, hBD5 and hBD9

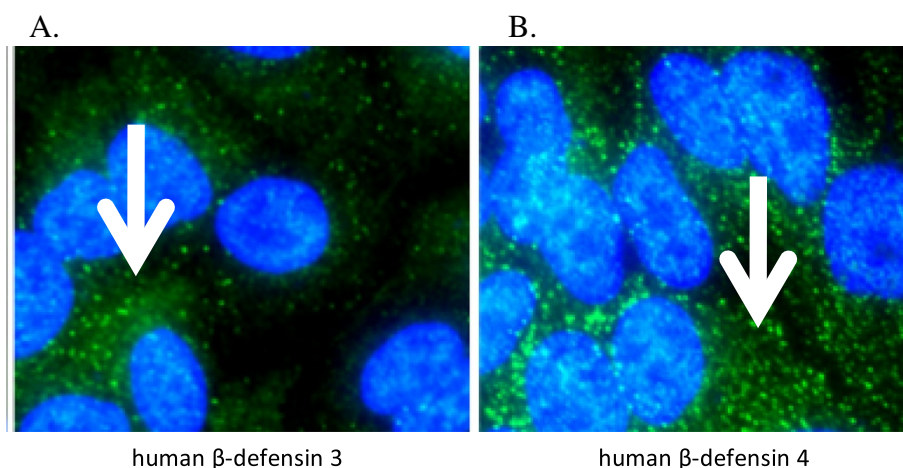
The human  $\beta$ -defensins hBD3, hBD5 and hBD9 were found to be significantly increased following treatment with 50  $\mu$ g/ml cigarette smoke extract (CSE) when compared to mock treated cells, and induced as much as with 10 ng/ml of IL-1 $\beta$  treatment. In response to CSE, hBD3 was 7.95 fold increased ( $p = 0.032$ ), hBD5 was 2.8 fold increased ( $p = 0.0083$ ) and hBD9 was 4.2 fold increased ( $p = 0.0078$ ). ( $n = 3$  for hBD3, 9,  $n = 6$  for hBD5) (Figure 1B).



### HBD8 and hBD18

Expression of the antimicrobial peptide gene hBD8 was not significantly induced in response to 50  $\mu$ g/ml cigarette smoke extract (CSE) (p > 0.05), however this  $\beta$ -defensin is significantly (p < 0.05) induced by 10 ng/ml of IL-1 $\beta$

treatment. The expression of another  $\beta$ -defensin peptide gene hBD18 was not statistically significantly induced with 50  $\mu$ g/ml cigarette smoke extract, although the means differed (Control = 1.000 $\pm$ 0.287, CSE = 3.207 $\pm$ 1.862) (p = 0.31). (Figure 1C).



**Figure 2 Immunofluorescence Microscopy of hBDs in A549 cells treated with cigarette smoke extract.** Green = human  $\beta$ -defensin (Alexafluor 488), Blue = nuclei (DAPI). **A.** hBD3 protein is observed in punctate staining in A549 cells (White arrow points to punctate staining). **B.** hBD4 protein is observed in heavily punctate staining in A549 cells (White arrow points to punctate staining).

### LL-37

Expression of the cathelicidin gene LL-37 was not significantly induced in response to 50  $\mu$ g/ml cigarette smoke extract (CSE) ( $p > 0.05$ ) either, however this antimicrobial peptide gene is significantly ( $p < 0.05$ ) induced by 10 ng/ml of IL-1 $\beta$  treatment. (Figure 1C).

### Visualization of hBDs by fluorescent microscopy

A549 cells exposed to 50  $\mu$ g/ml cigarette smoke (CSE) and processed *in situ* displayed positive staining for the internal presence of human  $\beta$ -defensins (Figure 2). Immunofluorescent microscopy was performed of cigarette-smoke treated A549 cells staining for hBD1, hBD3, and hBD4. Shown are representative images of staining for hBD3 and hBD4 peptides. No differential defensin staining was observed in the cells for hBD1 (data not shown), suggesting a similar result as was shown by the lack of induction of hBD1 seen in the qRT-PCR study.

### Discussion

A549 cells are considered to be a cell-line model for Type II alveolar epithelial cells, which secrete the lung surfactant and A549 express some of the markers of Type II cells [38,39]. In this study, we use the A549 cell model to measure relative gene expression of two classes of AMP; human  $\beta$ -defensins (hBDs) and the human cathelicidin (LL-37) in response to exposure to cigarette smoke extract. Total RNA was extracted from the samples and quantitative real-time PCR was performed to quantify AMP gene expression using gene specific primers.

The experimental approach was to expose A549 cells to 50  $\mu$ g/ml cigarette smoke extract (dissolved in DMSO), compared to control cells treated with DMSO alone. The positive control cells were exposed to 10 ng/ml

IL-1 $\beta$  which is known to up-regulate the expression of many defensin peptides [33]. The mRNA was then prepared from all samples, and qRT-PCR was performed for hBD1, hBD3, hBD5, hBD8, hBD9, hBd18, and the Cathelicidin LL-37. Overall, the application of 10 ng/ml IL-1 $\beta$  to A549 cells induced the expression of most of the tested human  $\beta$ -defensins (except hBD1, see below), confirming that the cells responded to the stimuli as expected.

We found that hBD3, hBD5, and hBD9 gene expression was up-regulated in A549 cells exposed to 50  $\mu$ g/ml CSE, while hBD1, hBD8, hBD18 and LL-37 gene expression did not significantly change. Expression of hBD1, hBD3 and hBD4 peptides within the A549 cells (*in situ*) was visualized by IF. This differential expression suggests that hBD3, hBD5, and hBD9 may play a role in the changes to the lung tissue observed in smokers. Establishing the differential human  $\beta$ -defensin gene expression following CSE treatment will add to our understanding of the molecular response of the lung alveolar epithelium to cigarette smoke exposure.

Our data demonstrates the differential induction of expression of specific  $\beta$ -defensin peptide genes as a result of exposure to cigarette smoke extract and suggests that three  $\beta$ -defensin genes (hBD3, hBD5, hBD9) may represent biomarkers of cigarette smoke extract exposure for alveolar epithelial cells [40-46]. These findings may contribute to a greater understanding of the role of the newly identified  $\beta$ -defensin peptides in the response of lung epithelial cells to cigarette-smoke. Further studies of other sample types from smokers, such as bronchoalveolar lavage could be used to validate these findings. Such biomarkers may have utility in studying the recovery of lung cells following the cessation of smoking.

Gene expression for hBD1 was not changed by exposure to cigarette smoke or IL-1 $\beta$ , as expected. The expression of hBD5, hBD8, hBD9 and hBD18, four of the new members of the human  $\beta$ -defensin gene family, are inducible by IL-1 $\beta$  in A549 lung epithelial cells, indicating they may respond to IL-1 $\beta$  regulation *in vivo* and may be involved in inflammatory responses and cell proliferation, differentiation, and apoptosis. Three genes, hBD8, hBD18 and the cathelicidin LL-37, were induced with IL-1 $\beta$  treatment, but not with cigarette smoke treatment, suggesting there is specificity of regulation for the different  $\beta$ -defensins. hBD3, hBD5, and hBD9 gene expression was increased by cigarette smoke extract treatment to the same extent as with IL-1 $\beta$  treatment. This suggests that these three  $\beta$ -defensins may have similar regulation by components of the cigarette smoke extract. Similarly, differentially-regulated expression of hBD3 and hBD9 was observed in infectious keratitis of the human eye [47] supporting that differential regulation of human  $\beta$ -defensin gene expression can occur. HBD3 is thought to be an important component of lung defense, such as in response to *Legionella* infection [48].

Exposure to cigarette smoke is thought to be harmful to host respiratory defenses through multiple modes of action [8]. Recent studies have suggested that cigarette smoke alters the production of hBD2 [28,29,49-54]. The induction of hBD2 and hBD3 expression (as well as IL-1B) in human gingival epithelial cells by cigarette smoke is thought to be through the activation of ERK1/2 and NF-kB pathways [30]. Nicotine has been reported to upregulate the expression of hBD2 via a p38 MAPK dependent pathway in human keratinocytes [55]. Cigarette smoke contains many toxic components, including potentially bacterial lipopolysaccharides [56], which in combination with the delivered nicotine could contribute to these observed effects of cigarette smoke. HBD2 is expressed in keratinocytes, the gingival mucosa and the tracheal epithelium [48,57-59] and alteration of its gene expression has been found in many diseases, such as infectious diseases, CF, and lupus erythematosus [60].

Xu recently showed that cigarette smoke extract significantly increased the production of IL-1 $\beta$  [57]. It was also shown that cigarette smoke inhibits the subsequent induction of hBD2 expression in response to bacterial lipopolysaccharide [52] or IL-1 $\beta$  [51], suggesting that cigarette smoke also may suppress the normal host defense response to bacterial pathogen exposure in the lung [35]. Kanda et al. recently demonstrated that exposure to hBD2 enhanced mRNA levels and secretion of many cytokines, including IL-1 $\beta$  [58]. Therefore, cigarette smoke may directly stimulate novel hBD production, leading to enhancement of IL-1 $\beta$ , which in turn could further stimulate additional hBD production, in a paracrine or autocrine loop. Interestingly, Kanda also reported that

the effects of hBD2 were counteracted by the drug Pentoxifylline (PTX) [58]. PTX is a drug with strong anti-inflammatory properties. This observation suggests that we should investigate the ability of PTX to potentially counteract the effect of chronic CSE-induced over-expression of human  $\beta$ -defensins.

Stimulation of oral squamous cell carcinoma (BHY-OSCC) cell line with hBD1 results in reduction of cell proliferation, whereas hBD2 and hBD3 stimulation causes promotion of cell proliferation, indicating that hBD2 and hBD3 might be protooncogenes in OSCCs [59]. Recent studies demonstrated all lung tumor samples, independent of their histological type, express hBD2 peptide, and its expression levels correlates with the differentiation grade of lung adenocarcinoma [61], suggesting that the role of human  $\beta$ -defensins in lung cancer should be further investigated also.

The health effects of cigarette smoke on lung health are significant. Establishing a link between smoking and the induction of novel and established antimicrobial peptides is an important step in understanding the pathology observed in cigarette smokers. AMPs may represent novel biomarkers of overall lung cell health status, as well as having potential direct effects in the pathogenesis of cigarette smoking. In the long term, we would like to investigate whether chronic antimicrobial peptide induction may contribute to lung pathology, which may occur before the occurrence of cancer or Chronic Obstructive Pulmonary Disease (COPD). If so, AMP induction could potentially be useful as both a biomarker of cigarette exposure, as well as a possible target for therapeutic intervention.

#### Abbreviations

CSE: Cigarette smoke extract; HBD: Human beta-defensin; BALF: Bronchiolar lavage fluid; TNF- $\alpha$ : Tumor necrosis factor alpha; TLR: Toll-like receptor; CF: Cystic fibrosis; COPD: Chronic obstructive pulmonary disease; PCR: Polymerase chain reaction; IF: Immunofluorescence; DMSO: Dimethyl sulfoxide; mRNA: messenger ribonucleic acid; DAPI: 4',6-diamidino-2-phenylindole; IL-1 $\beta$ : Interleukin-1 beta; LPS: Lipopolysaccharide.

#### Competing interest

The authors declare that they have no competing interest.

#### Authors' contributions

MVH conceived and designed the study. TP helped to design the study, performed key experiments and did data analysis. MVH and TP co-wrote the manuscript. SLP and DP performed some qRT-PCR experiments and contributed to the data analysis. All authors read and approved the final manuscript.

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